INFECTION AND IMMUNITY, Oct. 1997, p. 4322–4329 Vol. 65, No. 10
0019-9567/97/$04.00+0

Spiralin, a Mycoplasmal Membrane Lipoprotein, Induces T-Cell-Independent B-Cell Blastogenesis and Secretion of Proinflammatory Cytokines

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Received 21 March 1997/Returned for modification 23 May 1997/Accepted 21 July 1997

Mycoplasmas are bacteria which can cause respiratory, arthritic, and urogenital diseases. During the early phase of infection, mycoplasmas usually induce an inflammatory response and a humoral response preferentially directed against their membrane-bound, surface-exposed lipoproteins. In this report, we describe the effects on immune cells of spiralin, a well-characterized mycoplasmal lipoprotein. Purified spiralin stimulated the in vitro proliferation of human peripheral blood mononuclear cells and murine splenocytes. The stimulation pathway was probably different from that followed by *Escherichia coli* lipopolysaccharide because the effect of spiralin was not abolished by polymyxin B. Comparison of the effects of whole, native spiralin with those induced by proteinase K-digested spiralin or by the C-terminal half of spiralin (peptide $p[13.5]_T$) revealed that the first half of the protein, which contains the lipoylated N terminus, is responsible for the mitogenic activity. In contrast to whole spiralin, proteinase K-digested spiralin did not trigger murine B-cell differentiation and immunoglobulin G and M secretion. Stimulation of human or murine immune cells led to early secretion of proinflammatory cytokines (human tumor necrosis factor alpha and murine interleukin 1 or 6). Spiralin induced the T-cell-independent blastogenesis of murine B cells but did not stimulate T cells. Altogether, our data demonstrate that spiralin possesses potent immunostimulating activity, similar to that reported for lipoproteins of pathogenic gracilicutes (gram-negative eubacteria; e.g., *Borrelia burgdorferi* OspA and *E. coli* Braun lipoprotein), and are consistent with the fact that lipoproteins are major antigens during mycoplasma infections.

Mollicutes (trivial name, mycoplasmas) are the smallest self-replicating microorganisms. In humans and animals, these bacteria can cause respiratory, arthritic, and urogenital diseases. Moreover, several species are considered putative cofactors in the progression of various viral or bacterial diseases, notably, human immunodeficiency virus disease (for reviews, see references 5 and 20). This hypothesis is supported by numerous studies which have shown that mycoplasmas possess immunomodulating components involved in the activation or suppression of immune system functions (8, 30, 31, 33, 36).

In the early phase of mouse infection with *Mycoplasma pneumoniae* or *Mycoplasma pulmonis*, production of cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1), and IL-6, which are characteristic of an inflammatory response can be detected (10, 27). The absence of murein or lipopolysaccharide (LPS) endotoxin in mycoplasmas has prompted us to identify the constituents that mediate this inflammatory response. Lipoproteins containing an N-terminal glyceride-cysteine are major components of the cell membrane of mycoplasmas (38). Some of them, like spiralin, account for 20% or even more of the total membrane integral protein fraction (38). Several lipoproteins exhibit antigenic variation which would favor mycoplasmal escape from the immune defense and consequently contribute to the chronicity of infections (37, 39). Furthermore, being cell surface exposed, lipoproteins are likely to be preferentially targeted by the immune system. Indeed, the major antigens in the humoral response to *Mycoplasma penetrans* are two lipoproteins, p35 and p38 (12, 20), and the early humoral response to *Mycoplasma gallisepticum* is also directed against lipoproteins (15). Moreover, fractions containing lipoproteins from *Mycoplasma fermentans*, *M. penetrans*, and *Mycoplasma arginini* induce in vitro stimulation of immune cells, as evidenced by a proliferative response and/or secretion of cytokines (11, 14, 29, 30). The fractions used in these experiments consisted of Triton X-114 cell extracts named LAMPS or a phenol phase of delipidated membranes. As they were not composed of only lipoproteins, we cannot rule out the possible interference of other molecules (proteins, lipids, glycolipids, and detergent). Actually, the use of purified entities is necessary to clearly demonstrate the stimulatory effect of lipoproteins.

Hence, we have chosen to use the *Spiroplasma melliferum* spiralin to address this question. Indeed, spiralin is one of the most thoroughly characterized mycoplasma membrane proteins; its sequence has been determined (7), its acylation has been demonstrated (3, 13, 19, 41), and its topology has been elucidated (4). Furthermore, spiralin can be purified to homogeneity under nondenaturing conditions (40, 41). Unlike some other mycoplasmal lipoproteins, spiralin does not undergo antigenic variation.

The goal of this work was to answer the following questions. (i) Does spiralin have a mitogenic activity? (ii) Which part of the protein is responsible for this activity? (iii) Does spiralin induce the differentiation of splenocytes? (iv) Does spiralin

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trigger the secretion of proinflammatory cytokines? (v) What type of immune cell response is stimulated by spiralin?

MATERIALS AND METHODS

Reagents and suppliers. *N*-Tosyl-L-phenylalanyl chloromethyl ketone-trypsin, proteinase K, RPMI 1640, trypan blue, phytohemagglutinin A (PHA), concanavalin A (ConA), bovine serum albumin (BSA), and formalin were purchased from Sigma Chemical Co., St. Louis, Mo. Penicillin, streptomycin, and L-glutamine were from GIBCO BRL, Eragny, France. Fetal calf serum was from TechGen, Les Ulis, France. Tritiated thymidine (dThd) and HRP (horseradish peroxidase)-streptavidin were from Amersham, Les Ulis, France. *Escherichia coli* O5:B5 LPS and polymyxin B were from Difco Laboratories, Detroit, Mich. *Borrelia burgdorferi* OspA was kindly provided by L. Erdile, Pasteur Merieux Serum and Vaccines, Marcy l'Etoile, France. HRP-conjugated anti-immunoglobulin G (IgG) and anti-IgM and alkaline phosphatase-labeled anti-rabbit IgG were from Bio-Rad, Ivry sur Seine, France. $Pam_3CysSer(Lys)_4$-OH was obtained from Boehringer Mannheim, Meylan, France.

Bacterial culture and preparation of cell membranes. *S. melliferum* BC3 was grown as previously described (41). Cells were harvested by centrifugation at 15,000 × *g* for 1 h at 4°C and washed once in 50 mM phosphate-buffered saline (PBS; pH 7.4) containing 0.15 M NaCl and 8% sorbitol. The cells were then dispersed into 50 mM Tris-HCl buffer (pH 7.5) and disrupted by ultrasonication at 20 kHz twice for 1 min each time at 0°C. The membranes were centrifuged three times at 38,000 × *g* for 1 h at 4°C in 50 mM Tris-HCl buffer (pH 8.0) and finally depleted of the bulk of extrinsic proteins as described earlier (41).

Purification of spiralin and the $p[13.5]_T$ fragment. Spiralin was purified by agarose suspension electrophoresis after selective extraction with sodium deoxycholate (40, 41). Detergent-free spiralin micelles were obtained by extensive dialysis against Veronal buffer (pH 8.6; ionic strength, 0.015) for 36 h at 4°C and PBS (pH 7.5) for 12 h at room temperature (41).

Spiralin micelles were digested with trypsin as described previously (41). Briefly, 1 mg of detergent-free spiralin micelles was dispersed in 1 ml of 100 mM Tris-HCl (pH 8.0) and incubated for 24 h at 37°C in the presence of 3 mM NaN_3–1 mM $CaCl_2$–40 μg of *N*-tosyl-L-phenylalanyl chloromethyl ketone–trypsin. The preparation was then centrifuged at 260,000 × *g* for 15 min at 4°C to separate the water-soluble fraction from the insoluble one. The water-soluble fragment $p[13.5]_T$ was finally purified by size exclusion high-performance liquid chromatography with a Waters Protein-Pack 200SW column. The purity of spiralin and $p[13.5]_T$ was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (17) and silver staining of protein bands. Spiralin and $p[13.5]_T$ were kept at −80°C in PBS (pH 7.5) after irradiation (^{137}Cs, 15 min, 25,000 rads).

N-terminal sequencing of spiralin peptide. Purified $p[13.5]_T$ (50 μg) was subjected to SDS-PAGE (15 to 20% polyacrylamide linear gradient) (17) and electroblotted onto a polyvinylidene difluoride membrane in 50 mM Tris-50 mM boric acid buffer (pH 8.0). The $p[13.5]_T$ band was stained with 0.5% amido black 10B in methanol-acetic acid-water (40:1:59, vol/vol), excised, and submitted to Edman degradation. Sequencing was performed with an Applied Biosystems 470A protein sequencer with an on-line 120A phenylthiohydantoin analyzer.

Proteinase K digestion. Spiralin and $p[13.5]_T$ were totally digested with proteinase K for 2 h at 56°C (1 μg of proteinase K/2.5 μg of protein), and the protease was inactivated by heating for 10 min at 95°C. The efficacy of the digestion was checked by SDS-PAGE and silver staining.

Protein determination. Protein concentrations were determined by using the bicinchoninic acid method (Pierce, Rockford, Ill.) with BSA as the standard.

***Limulus* amoebocyte lysate assay.** The lack of endotoxin in spiralin preparations and in cell culture media was monitored by using the *Limulus* amoebocyte lysate assay as recommended by the manufacturer (BioWhittaker, Walkersville, Md.).

Purification and culture of PBMC and mouse spleen cells. Human peripheral blood mononuclear cells PBMC were obtained from healthy donors who had never been involved in work with spiralin or spiroplasmas. PBMC were isolated from fresh blood by centrifugation over Ficoll-Hypaque and cultured in RPMI 1640 supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, L-glutamine (2 mM), penicillin G (50 U/ml), and streptomycin (50 μg/ml) at 37°C under 5% CO_2 in a humidified atmosphere.

Seven-week-old female mice (BALB/c, C57BL/6, and C3H/HeOuJ) were purchased from IFFACREDO, L'Arbresle, France. Splenocytes were rapidly isolated from sacrificed mice. The erythrocytes were lysed with 0.87% NH_4Cl, and splenocytes were immediately washed twice with the culture medium. Spleen cells were then cultured under conditions identical to those used for PBMC and with the same medium supplemented with 0.05 mM 2-mercaptoethanol (2-ME).

Murine lymphocyte depletion. Depletion of the lymphocyte subset from freshly isolated murine splenocytes was performed by using immunomagnetic beads coated with Thy 1.2-specific monoclonal antibodies (MAbs) (Dynabeads mouse pan T; Dynal, Oslo, Norway) or B220-specific MAbs (Dynabeads mouse pan B; Dynal). The spleen cells were incubated for 20 min at 4°C with beads at a bead-to-cell ratio of 4:1. The efficacy of B- and T-cell depletion was checked by flow cytometry and reached 98.5 and 99.4%, respectively.

Proliferative response assay. Human PBMC ($0.75 \cdot 10^6$/ml) or mouse splenocytes (10^6/ml) were cultured in 96-well microtiter plates (Costar, Cambridge, Mass.) in 0.2-ml volumes with different concentrations of spiralin and $p[13.5]_T$ for various periods of time. Cell proliferation was measured after pulsing with 1 μCi of [^{3}H]dThd per well of the culture for 8 or 16 h. PHA (1 μg/ml), *E. coli* LPS (2.5 μg/ml), ConA (1 μg/ml), and *B. burgdorferi* OspA (0.5 μg/ml) were used as positive controls. Cell viability was checked by trypan blue exclusion. Each treatment was performed in triplicate. The results were expressed as average [^{3}H]dThd uptake (counts per minute in thousands). A Fisher test of variance comparison was performed to estimate whether the use of the *t* test for mean analysis was justified. In some cases, as indicated in the text, the modified *t* test was also used.

TNF-α, IL-1, and IL-6 titration. Human PBMC and BALB/c splenocytes (10^6/ml) were cultured in 24-well microtiter plates in 1-ml volumes with different concentrations of spiralin. After 1, 3, or 5 days, the culture supernatants were collected. TNF-α, IL-1, and/or IL-6 concentrations were determined by enzyme-linked immunosorbent assay (ELISA) in the supernatant (Quantikine, R&D SYSTEMS, Oxon, United Kingdom).

The J774 cell line (mouse BALB/c tumor monocytes-macrophages) was obtained from the European Animal and Cell Culture Collection and was cultured in the same medium as PBMC at a density of 10^6/ml before stimulation (37°C, 5% CO_2). J774 cells were stimulated with various agents (*E. coli* LPS, *B. burgdorferi* OspA, spiralin, proteinase K-digested spiralin, $p[13.5]_T$, and $Pam_3CysSer-Lys_4$-OH) for 24 h. Culture supernatants were collected, and cells were briefly sonicated at 0°C in the presence of 0.6 mM phenylmethylsulfonyl fluoride. Cell debris was eliminated by centrifugation (10,000 × *g*, 15 min, 4°C) before immunoassay of the total IL-1β in combined culture supernatants and cytoplasmic fractions.

Quantitation of secreted murine IgG and IgM. Secretion of IgG and IgM from stimulated murine splenocytes was measured by ELISA (25a). Briefly, anti-IgG and anti-IgM (100 and 500 ng/well, respectively) were used to coat the surface of 96-well microtiter plates in carbonate-bicarbonate buffer (pH 9.6) and incubated for 1 h at 37°C. The plates were washed three times and blocked for 1 h at room temperature with 1% BSA in PBS. After three washes, diluted culture supernatants or standard IgG or IgM solutions (0 to 50 and 0 to 300 ng/ml, respectively) were incubated for 1 h at 37°C in PBS containing 0.1% BSA and 10% fetal calf serum. Specific reactions were revealed by incubating the plates with HRP-conjugated anti-IgG or anti-IgM and using *o*-phenylenediamine as the chromogen. A_{492} and A_{620} were measured. Determinations were performed in triplicate, and the means are reported.

Cytofluorimetric analysis. Freshly isolated or 3-day cultured splenocytes were washed in PBS (pH 7.4) supplemented with 1% (wt/vol) BSA and 0.1% sodium azide (PBS-BSA-NaN_3). Cells were then incubated for 15 min at 4°C with conjugated MAbs specific for B-cell (rat anti-mouse CD40–R-phycoerythrin [RPE]) or T-cell (rat anti-mouse CD3-fluorescein isothiocyanate) (Serotec, Oxford, United Kingdom) markers and washed twice with PBS-BSA-NaN_3. Cells were fixed in 1% (vol/vol) formalin and applied to a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.). For each sample, data from 5,000 total splenocytes were collected in list mode, recorded on logarithmic scales, and analyzed with Lysis II software (Becton Dickinson). Morphological parameters (cell size and granularity) were used to quantify quiescent, blastic, and apoptotic cells. Quiescent cell morphology was the same as that observed ex vivo. Apoptotic cells were identified by their shrinkage, i.e., their decreased size, as previously described (18), and blast cells were identified as cells of increased size and granularity.

RESULTS

Purity of spiralin and $p[13.5]_T$. Spiralin was extracted from *S. melliferum* isolated membranes with two surfactants, Sarkosyl and sodium deoxycholate, and purified by preparative column electrophoresis. The purity of the protein was checked by SDS-PAGE and silver staining (Fig. 1A). Surfactant removal by extensive dialysis led to the formation of spiralin protein micelles displaying an average diameter of about 10 nm (Fig. 1B). The absence of contaminating exogenous bacterial endotoxin in spiralin preparations was monitored by the *Limulus* amoebocyte lysate assay and was found, as expected, to be lower than 0.1 endotoxin unit (EU)/ml, i.e., <0.01 ng/ml or <0.01 ng/mg of spiralin.

The $p[13.5]_T$ fragment was purified by high-performance size exclusion chromatography from a tryptic hydrolysate of spiralin as described in Materials and Methods. Its purity was verified by using SDS-PAGE (Fig. 1A). The N-terminal sequence of this fragment, HGEVTK, determined by the Edman technique, shows that its N terminus corresponds to histidine^{110} of the 219-residue spiralin polypeptide. As a molecular mass of 13.5 kDa corresponds to approximately 117 residues,

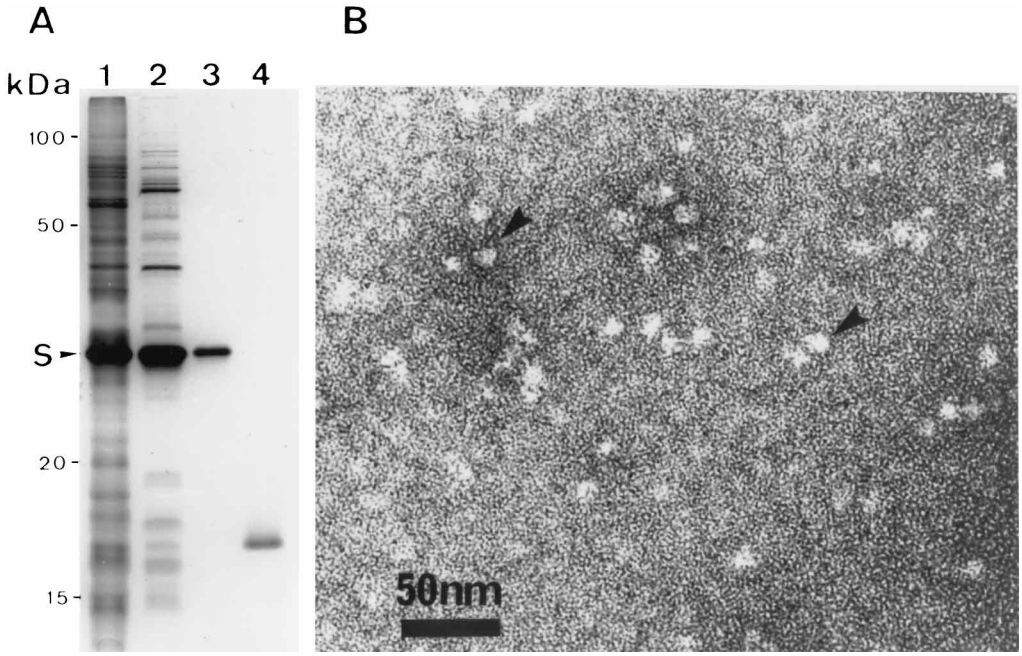

FIG. 1. SDS-PAGE analysis of purified spiralin and p[13.5]$_T$ (A) and transmission electron microscopy of spiralin protein micelles (B). (A) Lanes: 1, *S. melliferum* membrane proteins (15 μg) solubilized with 1% SDS; 2, membrane proteins (10 μg) selectively extracted with 100 mM sodium deoxycholate from the fraction insoluble in 20 mM Sarkosyl; 3, spiralin (1 μg); 4, p[13.5]$_T$ (1 μg). SDS-PAGE was performed with the discontinuous system buffer of Laemmli using a stacking gel (T, 4.8%; C, 2.6%) and a separating gel (T, 12.5%; C, 2.6%). Protein bands were revealed by silver staining. S, spiralin. (B) Micelles were stained with 2% uranyl acetate (pH 4.5) before observation. The arrowheads point out some micelle particles (diameter, 10 nm).

p[13.5]$_T$ is the second (i.e., C-terminal) half of spiralin. However, we cannot rule out the possibility that the terminal octapeptide VTATAPTE following lysine^{211} was chopped off by trypsin.

Spiralin micelle-induced human PBMC proliferation. Spiralin micelles induced the proliferation of PBMC from two of three human blood donors in a dose-dependent manner. After a 3.5-day stimulation, spiralin induced no detectable effect, but after 4.5 days, cell proliferation was detected (data not shown). At optimal concentrations, i.e., 3 to 6 μg/ml, spiralin induced a proliferation corresponding to 20% of that induced by PHA. When the protein was used at higher concentrations, it exhibited a toxic effect on the cultured cells, as evidenced by a decrease in dThd uptake.

Spiralin-stimulated TNF-α secretion by human PBMC. TNF-α secretion by human PBMC was determined in cell culture supernatant upon stimulation with various concentrations of spiralin (Table 1). Since the first day, TNF-α secretion was maximal and proportional to the spiralin concentration. In the presence of 6 μg of spiralin per ml, the secretion reached almost 70% of that induced by 1 μg of PHA per ml.

Spiralin-stimulated murine splenocyte proliferation. The PBMC response to spiralin stimulation varied from one donor to another. Consequently, the subsequent investigations were performed by using murine splenocytes to obtain more reproducible results. In this in vitro system, spiralin was mitogenic for BALB/c mouse splenocytes in a dose-dependent manner (Fig. 2). After 3 days of culture, cell proliferation was detected with 0.01 μg of spiralin per ml (by using the modified *t* test, $P < 0.01$) and the maximal dThd incorporation was observed with 5 μg of spiralin per ml. At this concentration, the incorporation levels induced by spiralin were 51, 31, and 184% of those induced by *E. coli* LPS, ConA, and *B. burgdorferi* lipoprotein OspA, respectively (Table 2). At concentrations higher than 12.5 μg/ml, spiralin was toxic for the splenocytes, as reflected by a marked decrease in the dThd incorporation level.

Spiralin also efficiently stimulated C3H/HeOuJ and C57BL/6 murine splenocytes ($P < 0.001$ for C57BL/6 mice and $P < 0.001$ for C3H/HeOuJ mice), but the proliferative response of the cells from BALB/c mice was the highest (Table 2).

To check the possibility that spiralin uses the same pathway of activation as *E. coli* LPS, spiralin and control molecules were pretreated before their addition to BALB/c splenocytes and incubation for 1 h with 10 μg of polymyxin B per ml, a concentration sufficient to neutralize the effect of 20 ng of LPS per ml. As previously shown (21), polymyxin B treatment abolished LPS activity but not that of *B. burgdorferi* OspA. Similar

TABLE 1. Induction of cytokine secretion by spiralin[a]

Cytokine[a]	Concn (pg/ml)							
	Medium		Spiralin		PHA		LPS	
	Day 1	Day 5	Day 1	Day 5	Day 1	Day 5	Day 1	Day 5
H-TNF-α	180	250	1,405	1,550	1,960	2,300	ND[b]	ND
M-IL-1β	8	13	58	60	ND	ND	16	29
M-IL-6	15	31	190	234	ND	ND	95	127

[a] H-TNF-α, TNF-α secreted by human PBMC; M-IL-1β and M-IL-6, IL-1β and IL-6, respectively, secreted by murine splenocytes. PHA and LPS were used at 1 μg/ml of culture medium. The spiralin concentrations were 6 μg/ml of human PBMC culture and 1 μg/ml of murine splenocyte culture. In experiments with human PBMC, an increase in the spiralin concentration from 1 to 6 μg/ml induced a proportional increase in TNF-α secretion, but in experiments with murine splenocytes, 1 μg of spiralin per ml induced maximal secretion. The cytokine concentrations shown are means of duplicate assays and are representative of two independent experiments.

[b] ND, not determined.

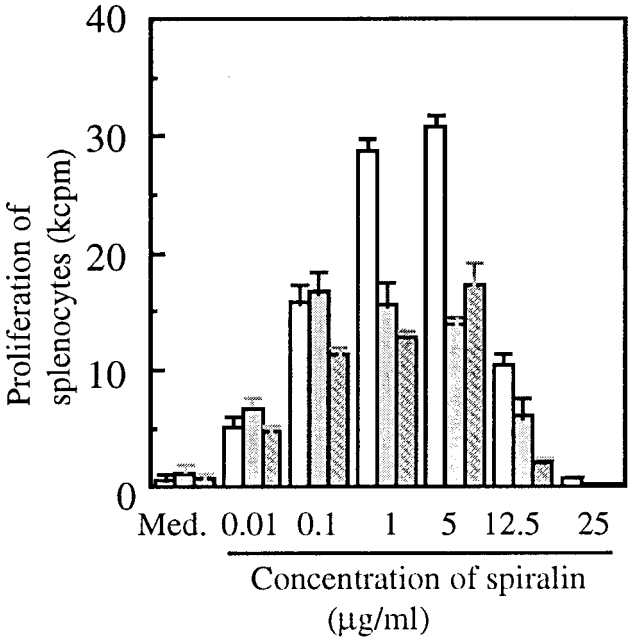

FIG. 2. Proliferative response of murine splenocytes to spiralin. Splenocytes (10^6/ml) were stimulated with various concentrations of spiralin. Splenocytes of BALB/c (□), C57BL/6 (▩), and C3H/HeOuJ (▨) mice were cultured for 3 days, and mitogenic activity was measured by determining the [^{3}H]dThd uptake of the cells. Med, medium. Data are means of three independent determinations.

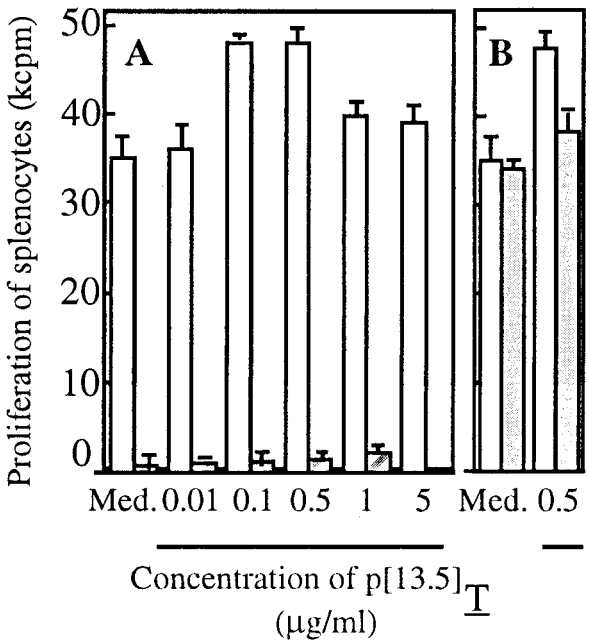

FIG. 3. (A) Proliferative response of BALB/c murine splenocytes induced by p[13.5]$_T$ with two different batches of fetal calf serum (batch 1, □; batch 2, ▨). (B) Proliferative response of splenocytes cultured with batch 1 of fetal calf serum stimulated by p[13.5]$_T$ (□) or by p[13.5]$_T$ digested with proteinase K (▨). In A and B, splenocytes (10^6/ml) were cultured and stimulated with various concentrations of p[13.5]$_T$. After 3 days, the mitogenic activity was measured as the [^{3}H]dThd uptake of the cells. Med., medium. Data are means of three independent determinations.

to the result obtained with OspA, the spiralin-induced proliferation was not significantly diminished ($P > 0.05$) (Table 2).

Proteinase K split spiralin into peptides which proved too small to be detected by SDS-PAGE (data not shown). This treatment reduced the mitogenic activity of spiralin on murine splenocytes by only 11% (Table 2). No activity was detected when heat-inactivated proteinase K was used.

p[13.5]$_T$-induced proliferation of preactivated murine splenocytes. Under the same conditions as those used to study the proliferation induced by spiralin, p[13.5]$_T$ was not mitogenic for BALB/c murine splenocytes (Fig. 3A). In contrast, when some batches of fetal calf serum were used for culture medium preparation, 0.1 and 0.5 μg of p[13.5]$_T$ per ml induced a low but significant level of [^{3}H]dThd uptake ($P < 0.05$). This weak activity was probably due to preactivation of the cells by the serum of the culture medium, as suggested by a high background level of dThd incorporation in the control samples (Fig. 3A).

However, even under optimal conditions, the mitogenic effect of p[13.5]$_T$ remained much weaker than that obtained with whole spiralin. The splenocyte proliferation was observed for concentrations of ≥0.05 μg of p[13.5]$_T$ per ml of culture medium ($P < 0.05$) and was maximal for 0.5 μg of p[13.5]$_T$ per ml. This activity was specific because it totally disappeared after the digestion of p[13.5]$_T$ with proteinase K ($P < 0.05$) (Fig. 3B).

Spiralin-triggered IL-1 and IL-6 secretion by murine cells. The ability of spiralin to stimulate the secretion of cytokines from splenocytes in the culture supernatants was tested with BALB/c mice because of their sensitivity to spiralin stimulation. Spiralin induced the secretion of the two proinflammatory cytokines tested, IL-1β and IL-6, by mouse splenocytes (Table 1). The level of cytokine secretion was independent of the spiralin concentration in the range of 1 to 6 μg/ml. The level of murine IL-1β secretion was maximal on the first day of stimulation and remained stable for 5 days. Murine IL-6 was also secreted on the first day of culture in the presence of spiralin

TABLE 2. Spiralin effect on murine splenocyte proliferation

Mouse strain	Mean ^{3}H uptake ± SD[a] (concn [μg/ml] of:)					
	LPS	ConA	OspA	Spiralin	Spi/pK[b]	Spi/pB[c]
BALB/c	60.0 ± 3.2 (2.5)	97.0 ± 4.1 (1.0)	16.5 ± 0.9 (0.5)	30.4 ± 2.1 (5.0)[d]	27.1 ± 1.6 (5.0)[d]	28.4 ± 1.7 (5.0)[d]
C57BL/6	70.5 ± 5.7 (2.5)	58.0 ± 2.8 (1.0)	38.5 ± 2.3 (0.5)	26.5 ± 1.9 (1.0)[d]	ND[e]	ND
C3H/HeOuJ	87.8 ± 3.9 (2.5)	89.0 ± 3.9 (1.0)	15.5 ± 1.1 (0.5)	16.0 ± 0.9 (1.0)[d]	ND	ND

[a] [^{3}H]dThd uptake is expressed as counts per minute (10^3), and the values are means of triplicate cultures. For untreated cells, the [^{3}H]dThd incorporation measured was about 1.5×10^3 cpm.
[b] Spi/pK, spiralin digested with proteinase K.
[c] Spi/pB, spiralin pretreated with polymyxin B.
[d] Spiralin concentration that induced the greatest [^{3}H]dThd incorporation.
[e] ND, not done.

TABLE 3. Induction of murine IL-1β synthesis by J774 cells[a]

Treatment	Murine IL-1β concn (pg/ml)	% of synthesis obtained with spiralin
Medium only (control)	19.5	6
LPS	291	90
OspA	213	66
Pam_3	185	57
Spiralin	322	100
Spi/pK	130	40
$p[13.5]_T$	7	2

[a] Several concentrations of each product were tested, and the amounts of murine IL-1β synthesized were dose dependent. The values shown were obtained with 0.5 μg/ml. A higher level of murine IL-1β production (303 pg/ml) was obtained with $Pam_3CysSer(Lys)_4$-OH at 0.1 μg/ml. This experiment was repeated three times and gave similar results. Pam_3, $Pam_3CysSer(Lys)_4$-OH; Spi/pK, spiralin digested with proteinase K. The murine IL-1β values are means of duplicate assays and are representative of two independent experiments.

but increased by about 23% after 5 days of culture. In the two cases, the amount of secreted cytokines was higher than that obtained with *E. coli* LPS.

To determine if the spiralin stimulation pathway involves macrophages and to compare the activity of spiralin with those of various stimuli in a homogeneous cell system, the mouse BALB/c J774 cell line was used and IL-1β production was determined after stimulation (Table 3). After a 24-h treatment, the concentrations of IL-1β induced by *E. coli* LPS, $Pam_3CysSer(Lys)_4$-OH, and *B. burgdorferi* OspA were 90, 57, and 66%, respectively, of that induced by spiralin. Under the same conditions, the production of IL-1β by cells stimulated by proteinase K-digested spiralin was decreased by 60% whereas $p[13.5]_T$ did not induce detectable IL-1β production (concentration, <5 pg/ml).

Spiralin-induced IgG and IgM secretion by BALB/c splenocytes. To determine the ability of spiralin to induce the differentiation of murine B cells into Ig-secretory cells, IgG and IgM in the cell culture supernatant were measured by ELISA after 1 and 5 days (Fig. 4A and B). In the presence of 1 μg of spiralin per ml, the levels of IgG and IgM production were similar and the kinetics were the same as those obtained with *E. coli* LPS treatment, i.e., no or very low secretion after 1 day and significant secretion of both Igs after 5 days of stimulation. The proteinase K-digested protein triggered neither IgG nor IgM secretion (after 5 days of culture, $P < 0.001$ and $P < 0.01$, respectively).

Spiralin-induced blastogenesis of BALB/c spleen B cells. To characterize the responsive cells, BALB/c splenocytes were cultured after adding spiralin, *E. coli* LPS, or ConA. After 3 days, cell morphology was analyzed by flow cytometry. Cells were labeled with CD40-RPE and CD3-fluorescein isothiocyanate MAbs and analyzed by gating size and granularity. Activated cells were identified by their increased size and granularity. Results indicated that 46% of the cells cultivated in medium alone died by apoptosis and only a fraction (14%) was activated (Fig. 5A). A majority of B cells were activated by LPS (89.5%) and spiralin (79%), whereas ConA stimulated mainly T cells (76%).

T-cell-independent B-cell response of BALB/c spleen cells to spiralin. To determine the type of spleen cells activated by spiralin, BALB/c splenocytes were depleted of B cells (Fig. 5C) or T cells (Fig. 5B) as described in Materials and Methods. By using this method, 98.5% of B cells and 99.4% of T cells were eliminated as determined by flow cytometry. These B-cell- or T-cell-depleted populations were then cultured for 3 days, and cell morphology was determined as described above.

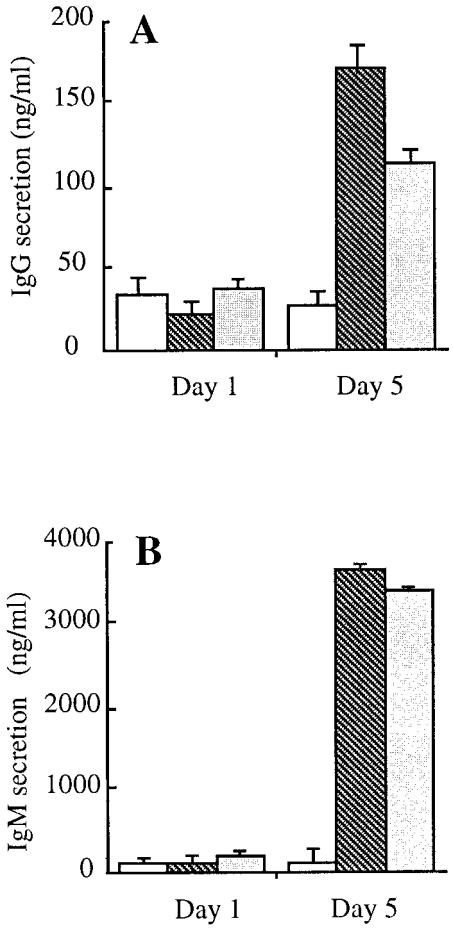

FIG. 4. Induction of IgG (A) or IgM (B) synthesis by spiralin. BALB/c murine splenocytes (10^6/ml) were unstimulated (□) or stimulated by 1 μg of *E. coli* LPS per ml (▨) or 1 μg of spiralin per ml (▩). Ig secretion was determined in the culture supernatant by ELISA after 1 and 5 days of culture.

When B-cell-depleted splenocytes were cultured in medium alone, the percentage of activated cells was very low (4%) (Fig. 5C). Although ConA was still able to stimulate cell proliferation, LPS and spiralin almost completely lost this activity (Fig. 5C). Indeed, flow cytometry analysis detected 55, 5, and 6% of blast cells with ConA, LPS, and spiralin, respectively. The residual activation obtained after stimulation with LPS or spiralin might be due to the proliferation and the blastogenesis of the small number of B cells not removed or to other spleen cells, such as NK cells (Fig. 5C).

After 3 days of stimulation of T-cell-depleted populations, significant activation was observed with LPS or spiralin whereas ConA was totally inefficient (Fig. 5B). Flow cytometry analysis revealed that the spiralin- or LPS-induced blast cells (70% of the population) were exclusively B cells. Moreover, the lipoprotein was able to stimulate a larger percentage of quiescent B cells than was LPS. Indeed, only 7% of quiescent cells remained after stimulation with spiralin versus almost 17% after LPS stimulation.

DISCUSSION

Lipoproteins constitute a class of membrane-bound proteins of particular interest in the study of bacterial pathogenicity. Indeed, they induce polyclonal activation of immune cells such

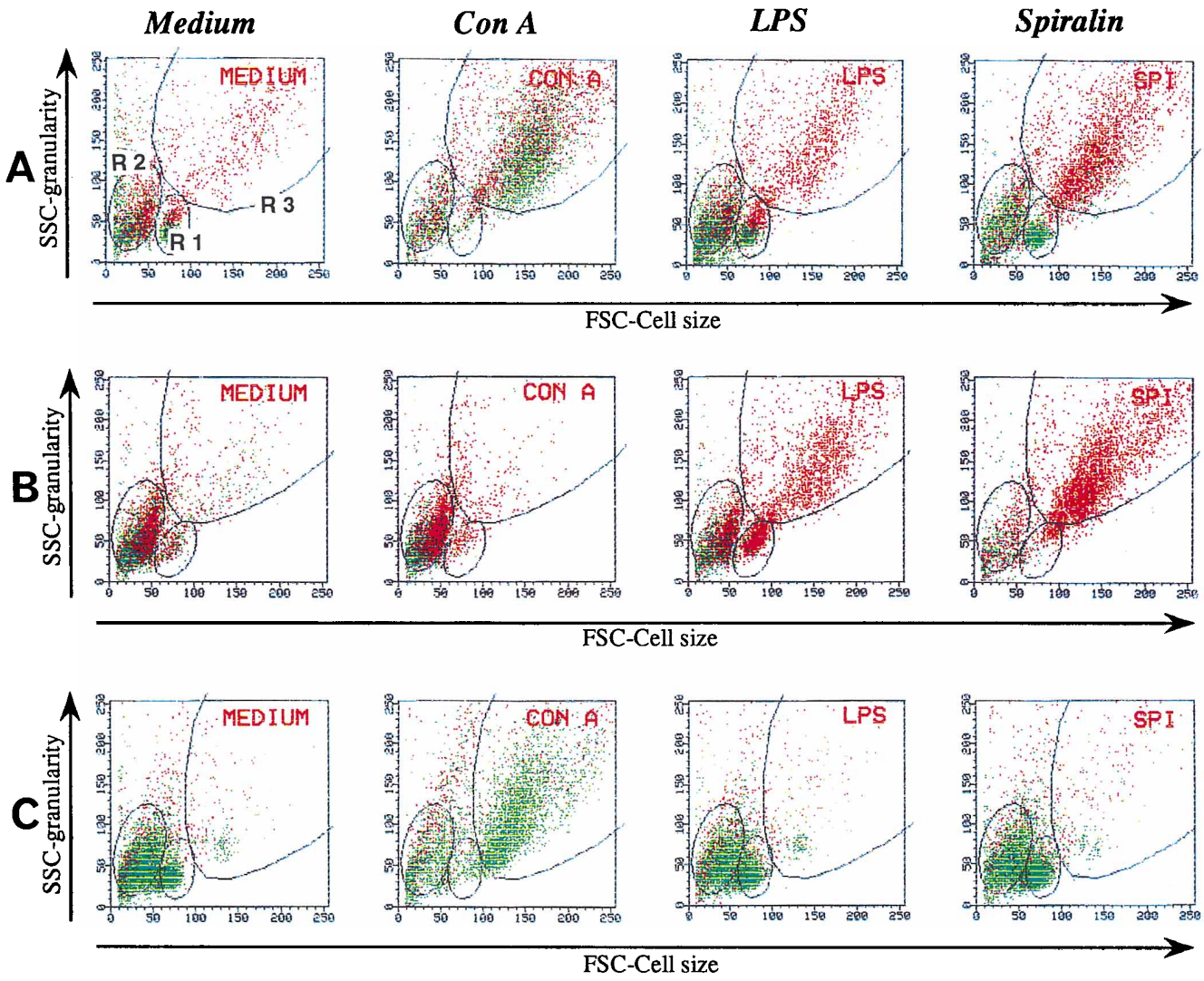

FIG. 5. Blast formation of lymphocytes induced by spiralin. Splenocytes stimulated with spiralin for 3 days were analyzed by flow cytometry by using dot plot analysis. Three-day cultured BALB/c splenocytes were labeled with anti-CD40–R-phycoerythrin (red dots) and anti-CD3-fluorescein isothiocyanate (green dots) and analyzed for size (forward scatter [FSC]) and granularity (side scatter [SSC]). ConA, LPS, and spiralin were added at concentrations of 1, 2.5, and 5 μg/ml. A, total splenocytes; B, T-cell-depleted splenocytes; C, B-cell-depleted splenocytes. R1, quiescent cells; R2, dead cells; R3, activated cells.

as monocytes-macrophage, B lymphocytes, platelets, and NK cells and their stimulating effects are evidenced by cell activation, cell proliferation, cytokine and nitric oxide production, induction of major histocompatibility complex expression, and immunoglobulin secretion (1, 28, 34, 35). Due to their immunogenicity and adjuvant potency, lipoproteins are currently being evaluated in vaccine preparations as purified antigens (16), synthetic lipopeptides (2), and recombinant lipoproteins (32) and produced by naked DNA vaccine (42). However, a major problem in studying the biological activities of mycoplasma lipoproteins comes from the difficulty in obtaining sufficient amounts of these compounds with a high degree of purity under nondenaturing conditions. This difficulty is overcome with spiralin, a very well-characterized mycoplasmal lipoprotein which is available in sufficient amounts in highly purified and native forms (Fig. 1).

Spiralin micelles devoid of detergent and lipids induced human PBMC and murine splenocyte proliferation, which is the first demonstration of the stimulating properties of a pure mycoplasmal lipoprotein for immune cells. Because the response of human PBMC varied from one donor to another, the murine system was chosen to obtain more homogeneous and reproducible data. Our results are consistent with previous studies which showed that bacterial lipoproteins from *E. coli*, *B. burgdorferi*, and *Treponema pallidum* activate immune cells (21, 22, 28). The kinetics of the induction of murine splenocyte proliferation by spiralin were the same as those of *B. burgdorferi* OspA or OspB (21) or *E. coli* Braun lipoprotein (22). Spiralin stimulated the proliferation of splenocytes at a lower level than LPS or ConA but similar to that induced by *B. burgdorferi* OspA (Table 2). Indeed, the range of spiralin activity was similar to that reported for other lipoproteins (from the least active to the most active): *E. coli* Braun lipoprotein (5 or 20 μg/ml, depending on experimental conditions) (22), spiralin (1 to 5 μg/ml, depending on the genotype of the mice) (Table 2), and *B. burgdorferi* OspB and OspA (0.5 to 1 μg/ml) (21). These different ranges of activity may be due to sequence or conformation diversity, in particular within the region in the

vicinity of the N-terminal glyceride cysteine. The fatty acyl composition of these lipoproteins may also be responsible for the observed differences. The latter interpretation is supported by the fact that synthetic lipotetrapeptides differing only in their fatty acyl chains display different mitogenic activities (34). In addition, an *M. fermentans* lipoprotein named MALP-2 has recently been described as being capable of activating macrophages in the picomolar range (25), compared to the 0.4 nM spiralin (or 0.01 μg/ml) necessary for induction of cell proliferation (Fig. 2). Determination of the acylated structure and the length of the N-terminal sequence allowing the highest activity is thus of the utmost importance to the understanding of the adjuvanticity of lipoproteins and for the development of lipoprotein- or lipoprotein-based vaccines.

Polymyxin B is a cationic cyclic lipopeptide known to inhibit LPS activity. This inhibition results from the binding of polymyxin B to the lipid A of LPS (24). As previously shown for *B. burgdorferi* OspA and OspB (21), polymyxin B treatment had no effect on the spiralin mitogenic effect, which was expected because of the very high purity of spiralin. This result also reinforces the conclusions of a study of the signalling events in immune cell activation by lipoproteins of *T. pallidum* and *B. burgdorferi*, lipoproteins and LPS (26). The authors demonstrated a difference in the cell surface initiating events and a convergence of the cellular responses of human monocytic cells stimulated by these various compounds, such as induction of the transcriptional activator NF-κB and cytokine production, which lead to an inflammatory response.

To locate the most active moiety of spiralin, we tested its properties after digestion with proteinase K. Interestingly, the mitogenic activity was only slightly reduced by proteolysis, which clearly demonstrates that the spiralin structural integrity is not crucial for the cell activation process in vitro. In accordance with this result, recombinant OspA with its N-terminal acylated end deleted loses its immunogenicity in mice, even in the presence of alum adjuvant (9), whereas synthetic analogs of the N termini of the *E. coli*, *B. burgdorferi*, and *T. pallidum* lipoproteins retain the stimulating properties of the whole lipoproteins, depending on the presence of the five or six amino acids next to the N-terminal acylated cysteine (28). Although the proteic part of spiralin is not sufficient to trigger cell activation, $p[13.5]_T$ is not totally devoid of immunological activity since it is immunogenic in rabbits (unpublished data) and contains, at least, a B-cell epitope within a 20-residue amphipathic segment (4) which is conserved in spiralins from different spiroplasma species (13). On the other hand, spiralin, but not proteinase K-digested spiralin, was capable of triggering the production by murine spleen cells of IgG and IgM as efficiently as *E. coli* LPS, indicating that spiralin was able to induce the differentiation of B cells (Fig. 4). Since T cells were not activated by spiralin (Fig. 5), this Ig secretion, like spiralin-induced blastogenesis, was T cell independent. Altogether, these results suggest a dual activity in spiralin: a nonspecific activation of immune cells by the lipoylated N end and a specific role of the polypeptide antigenic determinants in B-cell differentiation and Ig production. The nonspecific activity of the N-terminal part of spiralin is similar to the adjuvant potency of synthetic lipopeptides. This property is consistent with the higher level of rabbit humoral response against spiralin in comparison with other mycoplasma membrane antigens produced by immunized rabbits (40).

Mycoplasma pathogenesis has been associated with the early production of proinflammatory cytokines by their hosts, e.g., in *M. pneumoniae* and *M. pulmonis* infections of mice (10, 27). These multifunctional cytokines (TNF-α, IL-1, and IL-6) have a broad host-protective role in combating bacterial infection. In *M. pulmonis* infection, the ability of mice to resist disease has been correlated to the amount of TNF-α and IL-6 produced but not to a particular *H-2* or *Bcg* genotype, revealing the genetic complexity of the resistance to this mycoplasmal disease (6). These cytokines have also been implicated in the pathogenesis of syphilis and Lyme disease (21, 28). In addition, it has been shown in vitro that partially purified lipoproteins from *M. arginini* (14) and LAMPS from *M. penetrans* and *M. fermentans* (29) induce the production of these cytokines from human monocytes. Spiralin also triggered rapid in vitro secretion of human TNF-α, murine IL-1β, and murine IL-6 (Table 1), probably with a major contribution of monocytes-macrophages, as suggested by the experiment using the murine J774 cell line (Table 3). This activity was contained in the N-terminal part of spiralin but not in the C-terminal half, as evidenced by the lack of activity of $p[13.5]_T$ and the ability of proteinase K-digested spiralin to induce the production of IL-1β by J774 cells. This suggests that, among mycoplasmal membrane components, lipoproteins are likely the key inflammatory mediators during infection.

By using murine spleen cells depleted of B or T cells, we also found that, like *E. coli* LPS, spiralin induces T-cell-independent blastogenesis of B cells (Fig. 5) and is consequently a T-cell-independent antigen according to the nomenclature reviewed by Mond et al. (23). Under our experimental conditions, spiralin activated a larger population of quiescent cells than did LPS. Snapper et al. (35) demonstrated that bacterial lipoproteins and the lipopeptide $Pam_3CysSer(Lys)_4$-OH are not able to activate purified, resting murine B cells and require a costimulating molecule, such as a polysaccharide antigen, to induce cell proliferation and Ig secretion. Our study shows that T cells are not necessary for the cell response to spiralin stimulation, i.e., polyclonal activation and differentiation of B cells. Other cells, such as monocytes-macrophages and NK cells, could be involved in this process, but further studies are required to elucidate their specific contributions.

Our results obtained with spiralin support the assumption that natural mycoplasmal lipoproteins containing an N-terminal glyceride cysteine are potent B-cell polyclonal activators. This correlates with the strong and early humoral response directed against lipoproteins in some human or animal mycoplasmal infections (15, 20) and validates the interest in their use as antigens for serodiagnosis or for vaccine development.

ACKNOWLEDGMENTS

We are particularly grateful to Hervé Lecoeur for excellent technical assistance in flow cytometry and O. Neyrolles for the Ig ELISA. We also thank Yuko Sasaki and Sylvie Garcia for helpful discussion.

This work was supported by the Institut Pasteur, the CNRS, the Langlois Foundation (to H.W.), and a grant from the ANRS. C. Brenner was the recipient of a fellowship from the Association Claude Bernard.

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Editor: J. R. McGhee